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Métodos *in vitro* para o screening de biomateriais

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MÉTODOS *IN VITRO* PARA O SCREENING DE BIOMATERIAIS

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Engenharia Biomédica – Ramo Biomateriais, realizada sob a orientação científica da Doutora Paula Maria Façanha da Cruz Fresco, Professora Auxiliar da Faculdade de Farmácia da Universidade do Porto e do Doutor Mário Jorge Pereira, Professor Auxiliar, do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave *Biomaterial, Ensaios in vitro, Biocompatibilidade, Pseudokirchneriella subcapitata, Pandorina morum, Ecotoxicidade, Zircónia*

resumo

O presente trabalho consistiu numa revisão dos estudos efectuados até à data, no que respeita a metodologias *in vitro* para avaliação da biocompatibilidade de biomateriais, bem como prespectivas futuras. O aumento considerável na sua utilização, torna-os uma área de estudo atractiva, que sendo multidisciplinar, permite diversas abordagens. Sendo a biocompatibilidade a principal característica a mencionar num biomaterial, e das únicas a reunir consenso, foi sob este parâmetro que incidiu a primeira parte do trabalho, isto é, sob as diferentes metodologias *in vitro* que permitem a sua avaliação. Desta forma, foi analisada bibliografia nacional e internacional, pretendendo-se fazer uma revisão das metodologias que reunissem as melhores características (tendo em atenção aspectos laboratoriais como disponibilidade de material, duração dos ensaios, entre outros). Num segundo ponto pretendeu-se fazer uma abordagem dos biomateriais, usados actualmente em grande escala, mas sob um outro ponto de vista – impacto ambiental. Para isso, recorreu-se à realização de testes de ecotoxicidade, usando duas espécies de microalgas verdes – *Pseudokirchnereilla subcapitata* (Korshikov) Hindak e *Pandorina morum* (Müller) Bory. O biomaterial eleito foi a zircónia (ZrO_2). Ainda na mesma linha de investigação, e também pela escassez de informação observada durante a pesquisa bibliográfica, pretendeu-se compreender de que forma alterações no pH do meio, podem influenciar o grau de toxicidade. Estatisticamente, procedeu-se à análise dos dados obtidos recorrendo-se à análise de variância (ANOVA) de uma via, tendo sido aplicado o teste de Tukey, sempre que diferenças significativas foram encontradas. Esta análise permitiu verificar diferenças significativas no crescimento das duas algas, quando submetidas a concentrações crescentes do metal zircónio (Zr IV), observando-se ainda diferenças de sensiilidade apresentadas pelas mesmas, tendo *Pandorina morum* revelado maior sensibilidade. Concluiu-se ainda que variações no pH do meio alteram o grau de toxicidade do composto.

keywords *Biomaterial, In vitro assays, Biocompatibility, Pseudokirchneriella subcapitata, Pandorina morum, Ecotoxicity, Zirconia*

abstract The present work consisted in a review about the studies, untill today, surrounding the *in vitro* methodologies to evaluate biocompatibility, as well as future developments. The considerable increases in their utility, turns biomaterials in an interesting area of study, that being multidisciplinary, allows different approaches. Considering biocompatibility as a primordial characteristic in a biomaterial, and one of the only that get consensus among authors, this parameter as been choosen for the first part of the dissertation. According to this, national and international bibliography have been analysed, pretending to combine a set of the more effective methodologies, attending to laboratory aspects (such as material used and time dispense). In a second point, a different approach to biomaterial was consider, their environmental impact. For that, ecotoxicity tests were made, using to different species of green algae - *Pseudokirchneriella subcapitata* (Korshikov) Hindak and *Pandorina morum* (Müller) Bory. The biomaterial selected was zirconia (ZrO_2). The present study try to understand the influence of pH environmental alterations in the toxicity observed. Data analysis was done using variance analysis (one-way ANOVA), and Tukey test was applied when significant differences were found. Observing the results, significant differences on the growth of the two green algae were found, when submitted to crescent concentrations of zirconium (Zr IV), and it was also observed that they present different sensibilities to Zr (IV), with *Pandorina morum* showing more sensibility. Besides that, pH alterations influence compound toxicity, with alkaline environment increasing it.

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Capítulo I

Introdução geral

1. Introdução geral

Considerando o aumento gradual que se tem verificado no que respeita à aplicação de biomateriais, este é um tema vasto e de grande actualidade. Estes foram sem dúvida os factores chave para a escolha do tema da presente dissertação. Sendo um tema tão vasto, percebe-se facilmente a multidisciplinaridade que o caracteriza, motivo pelo qual se tornou necessário fazer uma selecção da área a abordar, permitindo desta forma uma pesquisa mais circunscrita e assim mais precisa. Por se revelar de grande importância, o estudo *in vitro* de biomateriais foi o tema visado. No entanto, e porque a panóplia de biomateriais é vastíssima, seleccionei dentro destes os biomateriais de aplicação dentária. O facto de estes serem utilizados com maior frequência levou-me a considerar que o seu estudo seria bastante pertinente, especialmente no que se refere a parâmetros pouco explorados, como o caso do seu impacto ambiental.

1.1. Conceito de biocompatibilidade

O estudo de biomateriais torna-se cada vez mais imperioso no quotidiano da sociedade. Devido à grande aplicabilidade que estes possuem, tornam-se peças integrantes e fundamentais de procedimentos médicos que ocorrem diariamente. No entanto, a sua aplicação nem sempre é bem sucedida, devido aos inúmeros mecanismos de resposta que o organismo possui e que são controlados por factores que envolvem as características do hospedeiro, do material e do próprio procedimento cirúrgico (Dee et al., 2002). De entre as possíveis reacções resultantes do contacto tecido-biomaterial podem salientar-se infecções (Arciola et al., 2004), citotoxicidade (Rogero et al., 2003; Moharamzadeh et al., 2006; Aranha et al., 2006), carcinogenecidade e mutagenecidade (Covacci et al., 1999).

O National Institutes of Health Consensus Development Conference define biomaterial como "qualquer substância (outra que não uma droga) ou combinação de substâncias, sintética ou de origem natural, que pode ser usada durante um período de tempo, como o todo ou como parte de um sistema que trata, aumenta ou substitui qualquer tecido, órgão ou função do corpo" (Williams, 1987). Apesar das várias definições, há contudo um parâmetro comum, já que todos os autores reconhecem o biomaterial como uma classe à parte de todos os outros materais, pelo facto de a biocompatibilidade ser uma característica obrigatória (Kirkpatrick et al., 2005). É este o requisito principal que um biomaterial deve respeitar, em última análise, não obstante o cumprimento de muitos

outros requisitos a que um biomaterial deve obedecer. Por este motivo torna-se fundamental compreender o conceito para perceber a sua real importância. No passado não eram realizados estudos directamente relacionados com o uso de biomateriais, embora a sua aplicação já existisse. Em vez destes, recorria-se a dispositivos fabricados a partir de materiais que eram projectados para servir diversas necessidades industriais, sendo o grande volume destinado a aplicações aeroespaciais. Estes dispositivos eram testados em corpos de animais e humanos. Geralmente, estes testes resultavam em conclusões confusas e sem grande aplicabilidade prática (Dee et al., 2002). Para prever, dentro dos possíveis, a ocorrência de reacções adversas ao biomaterial implantado, é necessário garantir a sua biocompatibilidade, recorrendo-se para o efeito a metodologias que permitem avaliar a biocompatiblidade do material e de alguma forma prever a reação do organismo ao mesmo (Hanks et al., 1996; Kirkpatrick et al., 2005). Apesar da grande importância que estas metodologias *in vitro* representam na avaliação da biocompatibilidade dos biomateriais, a sua utilização deve ser feita com algumas reservas. Estudos anteriores demonstraram já situações nas quais um biomaterial dentário (óxido de zinco e eugenol), caracterizado como tóxico por estes testes, quando usado em aplicações clínicas revelou-se eficaz e foi utilizado com sucesso (Schmalz, 1997).

A palavra biocompatibilidade, parece simples de compreender, no entanto a compreensão do conceito estende-se muito para além da interpretação da palavra. Segundo Williams (1998) qualquer biomaterial ou dispositivo médico implantado, não deve causar qualquer reacção adversa ao hospedeiro que o vai conter, no entanto, e embora os requisitos imponham total inércia por parte do biomaterial no hospedeiro, nenhum material implantado em tecidos vivos é completamente inerte; todos os materiais provocam uma resposta por parte do tecido hospedeiro (Hench and Wilson, 1993; Wataha, 2001). O aparecimento de novos biomateriais resulta da fusão de conhecimentos provenientes das mais diversas áreas, que passam pela mecânica, engenharia dos tecidos ou materiais. Destes estudos complexos resultam novos biomateriais com características cada vez mais semelhantes às estruturas que pretendem substituir/mimetizar no organismo, pretendendo-se desta forma garantir a sua biocompatibilidade. O seu estudo é um tema muito complexo e de difícil consenso, entre outros, pelo facto de justapor ciências tão distintas como materiais, mecânica e biologia (Williams, 1998). De uma forma geral, podem considerar-se dois aspectos principais no que respeita à definição de biocompatibilidade; um é a ausência

de efeitos de citotoxicidade, sendo que o outro aspecto a considerar é a biofuncionalidade do dispositivo implantado (Kirkpatrick et al., 2005). Isto significa que a escolha do material mais indicado para um deteminado dispositivo médico, implica uma noção prévia do local onde vai ser usado, bem como as características do meio biológico ao qual estará exposto (Dee et al., 2002); especialmente pelo facto de o material afectar o hospedeiro, por um lado, e por outro ser simultaneamente afectado pelo mesmo (Wataha, 2001). A citotoxicidade representa um papel de extrema relevância no que respeita ao estudo da biocompatibilidade de um dispositvo médico (ISO 10993), sendo a sua avaliação obrigatória para qualquer biomaterial antes de qualquer aplicação clínica e, podendo em muitas situações funcionar como elemento eliminatório do material em estudo. Ou seja, no caso de resultados positivos no que respeita à citotoxicidade do biomaterial *in vitro*, há geralmente rejeição do material, embora haja excepções já verificadas (Schmalz, 1997).

1.2. Ecotoxicidade de biomateriais

Embora seja inegável a importância da aplicação de biomateriais, é um facto que por si só não justifica que sejam postas de parte preocupações ambientais. Assim, pretendeu-se analisar qual o impacto ambiental relativamente à aplicação destes biomateriais. A ausência de bibliografia foi um dos motivos que levaram à realização deste estudo, pretendendo-se desta forma chamar a atenção para o problema e suscitar a realização de novos testes nesta área.

O aumento da produção de resíduos nocivos, implicam uma medição quantitativa do seu impacto ambiental, sendo que os testes de ecotoxicidade são uma das formas encontradas para o conseguir, medindo para tal o impacto dos resíduos em organismos vivos (Fuentes et al., 2006). Contaminantes, como metais, desenvolvem um papel importante no ambiente aquático quando interferem com os ecossistemas, colocando em perigo recursos, como a água para consumo. Substâncias indiscriminadamente drenadas para sistemas aquáticos, podem ser adsorvidas às plantas, animais e matéria orgânica, ou absorvidas por organismos aquáticos (Dankwardt et al., 1998). No entanto, na análise do efeito tóxico provovado por poluentes, como o zircónio (representado por Zr (IV) daqui em diante), deve ter-se em atenção as características do meio, já que são inúmeros os factores susceptíveis de causar alterações na espécie metálica, sendo que a toxicidade daquelas depende do tipo de metal, da sua concentração, do pH do meio, do potencial redox, da

temperatura, do conteúdo iónico, da matéria orgânica e da luz, que influenciam a forma química do metal e consequentemente a sua disponibilidade (de Filippis and Pallagly, 1994; Jjemba, 2002; Starodub et al, 1987; Thiele-Bruhn, 2003; Tolls, 2001). Assim, a biodisponbilidade e toxicidade do metal, são controladas pela especiação do metal na água (Meylan et al., 2003). A retenção de alguns metais por microalgas é sensível a alterações de pH (Boullemant et al., 2004; Martínez and McBride, 2001). O efeito dos poluentes em ambiente aquático não pode ser avaliado de forma adequada considerando apenas parâmetros físico-químicos; desta forma, existem várias metodologias baseadas na aplicação de indicadores biológicos (Maciorowski et al., 1981), aceites por organizações como a American Public Health Association, a American Water Works Association e a Water Pollution Control Federation. No entanto, a escolha do organismo mais adequado a usar não é consensual. A toxicidade de uma sustância é geralmente medida usando métodos padronizados de inibição de crescimento algal, utilizando para o efeito espécies padrão (ASTM, 2002; OECD, 2002; USEPA, 2002). Foram assim realizados testes de ecotoxicidade, utilizando duas algas verdes – *Pseudokirchneriella subcapitata* (Korshikov) Hindak e *Pandorina morum* (Müller) Bory, pertencentes à classe Chlorophyceae. A sua escolha seguiu os critérios estabelecidos na literatura (ASTM, 2002; OECD, 2002; USEPA, 2002), que recomendam *Pseudokirchneriella subcapitata* (Korshikov) Hindak na realização de testes de inibição de crescimento algal (ASTM, 2002; OECD, 2002; USEPA, 2002; Pereira et al., 2005). Desta forma, e por ser relevante o seu estudo, estudar-se-á simultaneamente a influência de diferentes valores de pH na toxicidade do Zr (IV).

1.3. Zircónia como Biomaterial

O biomaterial escolhido foi a zircónia (ZrO_2). No passado era conhecido apenas o zircónio (Zr IV) como uma gema. Este é um elemento químico (Zr), cinzento claro, lustroso e muito resistente à corrosão, é mais leve que o aço e a sua dureza é semelhante à do cobre. A sua principal fonte é o silicato de zircónio (ou zircão), que pode ser encontrado em depósitos localizados na Austrália, Brasil, Índia, Rússia e Estados Unidos. De entre os elementos de transição, apenas o Fe, Ti and Mn são mais abundantes que o zircónio, que por sua vez prefaz 0.016%, 162 ppm das rochas crustais (Greenwood and Earnshaw, 1984; Hulbert, 1993; Li and Hastings, 1998). A zircónia é o dióxido deste metal, e foi identificada em 1789 pelo químico alemão Martin Heinrich Klaproth (Piconi and

Maccauro, 1999). A sua escolha deve-se ao aumento significativo que se tem vindo a verificar na aplicação deste biomaterial, principalmente pelas características que o tornam tão atractivo enquanto cerâmico bionerte, destacando-se a sua biocompatibilidade excepcional, estabilidade mecânica, resistência à biodegradação e ao desgaste, bem como a estabilidade química em ambiente fisiológico (Hulbert, 1993). No entanto, existem também alguns problemas na sua aplicação, sendo o principal a considerar a sua radioactividade. O tório e o urânio são elementos radioactivos que se encontram frequentemente na companhia da zircónia, tornando-se extremamente dispendioso proceder à sua separação (Hulbert, 1993). Trata-se de um problema ainda pouco explorado e por isso pouco esclarecido. Especialmente no que se refere a implantes que se destinam a colocações em locais visíveis, a importância estética que estes assumem torna-se preponderante na escolha do material mais adequado. Desta forma, e no caso concreto dos implantes dentários, características como a cor do biomaterial são também um factor a considerar. Atendendo a este aspecto, também aqui a zirconia representa uma mais valia. A questão estética assume sem dúvida um papel primordial no caso particular dos implantes dentários, tendo em conta que o sorriso é a nossa primeira apresentação perante a sociedade; por este motivo os cuidados a ter com a saúde dentária têm vindo a assumir um papel cada vez mais relevante, suscitando aos profissionais a necessidade de corresponder às expectativas criadas pelos seus pacientes. O médico dentista tem neste processo um papel fundamental, no entanto, para que a colocação do implante seja possível e bem sucedida, existe por trás uma vasta equipa de profissionais. Considerando o facto de a colocação de próteses, no geral, mas concretamente próteses dentárias, ser actualmente considerado um processo simples, a sua aplicação tornou-se um acto rotineiro em qualquer consultório dentário. Daqui resulta uma quantidade considerável de resíduos provenientes dos mais variados biomaterias usados, nomeadamente de metais.

2. Objectivos

Pretende-se realizar uma revisão relativamente aos estudos realizados até à data sobre ensaios de biocompatibilidade *in vitro*, bem como perspectivas futuras. É objectivo desta análise, reunir um conjunto de metodologias eficazes na avaliação da biocompatibilidade de novos biomateriais, que surgem cada vez com maior frequência, e que exigem ser testados *in vitro* para que possam ser utilizados no corpo humano. Este trabalho pretende, através da análise cuidada de bibliografia nacional e internacional, indicar um método que apresente vantagens comparativamente com os restantes, tendo em consideração que esta escolha envolve inúmeros factores, todos eles relevantes em laboratório, nomeadamente o tempo dispendido no procedimento, o material necessário à sua consecução e os custos que este envolve.

Estudos de ecotoxicidade tornam-se cada vez mais relevantes tendo em conta a sociedade em que vivemos. Desta forma, pretende-se compreender de que forma a evolução na área dos biomateriais pode influenciar o meio ambiente. Apesar de um vasto leque de biomateriais, a escolha recaiu sobre a zircónia (ZrO_2) devido ao aumento significativo na sua aplicação. Assim, utilizando 2 algas verdes, recomendadas para testes de ecotoxicidade, procedeu-se à análise do efeito tóxico do composto em estudo. Visto que existe um aumento significativo na utilização deste tipo de materiais, e que se verifica uma lacuna na bibliografia no que a este tema diz respeito, é preponderante perceber de que forma ele poderá vir afectar o meio ambiente e a nossa qualidade de vida.

Desta forma, pretende-se alcançar alguns objectivos concretos neste trabalho:

- Analisar, de uma forma geral, as metodologias *in vitro* utilizados no estudo da biocompatibilidade de materiais com aplicação em próteses dentárias;
- Concluir relativamente à metodologia mais eficaz;
- Avaliar o efeito tóxico do biomaterial no crescimento algal;
- Utilizar o metal zircónio para garantir a presença das concentrações que se pretende testar;
- Avaliar a influência do pH na toxicidade do biomaterial;
- Calcular os valores de CI_{50} para as duas espécies de microalgas escolhidas.

Pelo facto de a zircónia (ZrO_2) se tratar de um cerâmico insolúvel, houve necessidade de encontrar um composto que disponibilizasse o metal pretendido – Zr (IV), por um lado, e por outro que fosse solúvel nas condições pretendidas para a realização do teste de

ecotoxicidade. Assim, o composto usado foi o nitrato de zirconil - $ZrO(NO_3)_2.xH_2O$ (Sigma-Aldrich, 380679 18411BB), que é uma fonte de zircónio muito hidrosolúvel. Por forma a garantir que o efeito tóxico resultaria apenas da acção do zircónio, realizaram-se testes preliminares na presença da mesma quantidade de nitratos presente no composto, para se verificar que estes não influenciam o crescimento algal.

3. Estrutura da dissertação

A presente dissertação apresenta-se composta por 4 secções. O Capítulo I pretende contextualizar o trabalho apresentado, consistindo em 3 subpontos relevantes para a percepção do desenvolvimento da dissertação. Assim, foi feita uma abordagem sobre aquele que será o conceito fulcral nos capítulos seguintes, a biocompatibilidade, esclarecendo a sua importância enquanto característica fundamental que é de um qualquer biomaterial, considerando o seu contacto directo com o interior do organismo. Num segundo subponto, procedeu-se a uma breve menção relativamente aos ensaios de ecotoxicidade, já que o trabalho prático que é parte integrante desta dissertação, consiste precisamente na elaboração de testes de ecotoxicidade. Por fim, procedeu-se à classificação e caracterização do biomaterial escolhido. Na mesma secção são apresentados os objectivos traçados inicialmente para a elaboração desta dissertação. O Capítulo II consiste num artigo de revisão, submetido à revista *Dental Materials* com o qual se pretende conseguir uma perspectiva geral dos estudos realizados até à data, no que respeita a metodologias *in vitro* aplicadas à avaliação da biocompatibilidade de materiais, mas também concluir acerca daquela(s) que possa(m) ser mais eficaz(es). Perspectivas do que o futuro poderá trazer para área em estudo são também alvo de uma pequena análise. O Capítulo III é constituído por um artigo submetido à revista *Fresenius Environmental Bulletin*, descrevendo o estudo efectuado relativo à ecotoxicidade do Zr (IV) no crecimento de duas algas verdes. No Capítulo IV é realizada uma discussão geral dos resultados obtidos no trabalho.

Capítulo II

In vitro biocompatiility of dental materials - A mini-review

In vitro biocompatibility of dental materials – A mini-review

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Abstract

The increasing use of biomaterials in dentistry requires great performances from the methodologies used to test their biocompatibility. Thus, considering the importance they rule in prosthetic procedures, this paper pretends to review and analyse current knowledge about test systems applied to the study of safety and efficacy of new medical devices. To understand the biocompatibility concept turns fundamental to analyse the whole situation of prosthesis placement, as well as the understand of biocompatibility as a dynamic process. Attending to the fact that medical devices will be used inside the organism, it becomes fundamental to understand the diversity of reactions to the presence of the biomaterial, as well as the cells involved in those reactions. This kind of knowledge lead us understand the different types of testing systems and principles for *in vitro* assays. Tissue engineering may be the future to avoid test animals and allow conclusions from tissue culture similar to those obtained from *in vivo* tests. By now, the main obstacle to transpose is the fact that cultures are protected from the defense mechanisms that assists cells within the body.

Keywords: *In vitro* assays; Methodologies; Biocompatibility; Cytotoxicity; Inflammatory response; Dental Materials; Cell types; Stem cells; Co-cultures; 3D cultures.

1. Biocompatibility concept

Biomaterials are used nowadays, several times per day in every dentary clinic, so it turns primordial to guarantee the efficacy of those medical devices. Biocompatibility is the most important requirement to a biomaterial, and it has to be previously established and approved by regulatory agencies, like the Food and Drug Administration (FDA) in the United States of America, or internatonal standards compiled as ISO 10993 (ISO, 1998). Biocompatibility is not a very consensual concept, and maybe the cause is the great number of sciences involved at the same time (Williams, 1998). Although, there are 2 parameters about every researchers are in accordance: the nonexistence of cytotocixity and the biofunctionality of medical devices (Kirkpatrick et al., 2005). ESB Consensus Conference I define biocompatibility as "the ability of a material to perform with an appropriate host response in a specific application" (Williams, 1987). It's obvious that any kind of test must take in consideration the place and function that the medical device is going to develop (Dee et al., 2002). So, the previous known about the exactly place and function the biomaterial is going to take within the body, is fundamental to the choice of the most adequate methodologie to test the biocompatibility, allowing the researcher to understand the kind of cells involved, as well as the biological environment (Kirkpatrick et al., 2005). To build the concept even more complex, must be consider the dynamics that characterize biocompatibility, because the daily changes in the body may not be forget, as the consequences in the biomaterial placed within (Wataha, 2001).

Biocompatibility testing, in the present, includes different concerns, like rapid and low cost methodologies, and always as possible pass up animals (Cruz et al., 1998); pretending at the same time, the most similar *in vivo* situation in *in vitro* methodologies (Schmalz, 1997). For that, a great contribute come from tissue engineering and their cell cultures.

2. Established testing systems

The standardization is fundamental to allow comparisons between different studies, what would be impossible if there were no standard procedures. International Standard Organization established a set of guidelines to assure the safety of medical devices; ISO 10993, with 18 Parts, is responsible to establishe the necessary tests for medical devices. *In vitro* cytotoxicity assay is the first test to evaluate the biocompatibility of any kind of material for medical device usage, recognized in 1987 by Organization for Economic Cooperation and Development (OECD), and in 1993 by Food and Drug Administration (Cruz et al., 1998). ISO 10993-5 established a battery of tests, whose choice will depend on the material, the function and place were its gone be placed. Only after prove the total absence of cytotoxicity the biocompatibility study proceeds. Cytotoxicity tests are widely recommended because of their characteristics, such as a rapid evaluation, standardized protocols and the obtained results, quantitative in one hand and on the other the possibility of comparison with other data. These tests require cell cultures, which allow to followup changings in their function; they could be used both continuous lines and primary cultures (Cenni et al., 1999). Continous lines are more reproducible but have great chances of loosing their phenotype. Primary cultury possess a very intense variability (result from individual characterisics) therefore are closer to the clinical situation (Kirkpatrick et al., 2005; Schmalz, 1997). Otherwise, cell cultures present more sensitivity to toxic effect of biomaterial than observed in *in vivo* environment (Cenni et al., 1999). Previous studies have shown a situation in which a dental material was characterized has a toxic in *in vitro* assays, and then used successfully in clinical applications (Schmalz, 1997). Thus, is fundamental analyse carefully each situation, and be conscious about the differences between *in vitro* and *in vivo* environments during extrapolation. In spite of that, cell culture allows, as far as possible, simulate the clinical situation. Cell culture development follow established guidelines of *in vitro* growing (ISO 10993-5). To get *in vitro* tests as close as possible to the clinical situation, a new concept was introduced, considering the case of dental restorative materials, and refers to the require of a barrier located between the material tested and the target cell (Hanks et al., 1996), developed on 1974 by Outhwaite et al.. This barrier proves to get results more similar to those obtained *in*

vivo (Wataha, 2001). This device pretends to simulate the dentin layer; dentin was presented to be used as a barrier between cells and the material tested (Tyas, 1977; Meryon, 1984; Hanks et al., 1996), by using human dentin cut in slices or as dentin pressed chips, but difficults are felt in the uptake of human dentin. The use of bovine dentin disks was the solution found (Schmalz et al., 1994).

3. Principles of biocompatibility assays *in vitro*

Biocompatibility tests intend to be low-cost and valuable methods, avoiding, as much as possible, the use of animals (Schmalz, 1997). ISO 10993 recomends the *in vitro* cytotoxicity assay as the first test to evaluate the biocompatibility of any kind of material for medical device usage (ISO, 1998). Cytotoxicity tests represent the initial stage of study biocompatibility medical device, and are used to detect toxic effects of those in the cells – death cell or nocive effect in the cellular functions (Malmonge et al., 1999). The biological systems used for screening toxicity are cell cultures (Polyzois, 1994). *In vitro* biocompatibility tests occur in a test tube or in cell-culture dish, what mean they have to happen externall to living organism; the general draw is to put cells contacting with the material (Wataha, 2001). Using the same type of cells that are going to be in contact with the biomaterial within organism, allow predicting reactions from this one (Cenni, 1999). For cytotoxicity evaluation, ISO 10993-5 "Biological Testing of Medical Devices – Part 5: Tests for Cytotoxicity – in vitro methods" is recommended, but although the principal factors are determined for these methodologies, they are not totally strict, having some decisions to the researcher, like the choice of the cell type, the duration of the test or the method to quantify the results (Harmand, 1997). There are benefits about cytotoxicity test that make them one of the most used in biocompatibility studies, namely their quickly, low cost and sensitivity. The last one can be explained with the fact of cells being totally isolated from all the protective mechanisms placed in the organism (ISO, 1998). Cytotoxicity can be determined by qualitative or quantitative evaluation (Hornez et al., 2002; Rogero et al., 2003). Many cytotoxicity studies point to the toxic evaluation of a single component, but its important attend to the combination of different components, because they act together; in this situation, 3 effects could be observed: additive, synergistic or antagonistic (Hanks et al., 1996).

4. Cell types for biocompatibility assays *in vitro*

The choice of the most indicated cell type depend on the application that the medical device is going to serve (Kirkpatrick et al., 2005), thus are employed the same type of cells that would be in contact with the biomaterial *in vivo* (Cenni et al., 1999). *In vitro* methodologies using cellular cultures are succefully used because of their reproductibility, quickly, sensibility and low cost procedures to study biocompatibility (Rogero et al., 2003). Changes in cell functions, caused by the interaction with biomaterials, can be observed and measured if cell cultures were used, with both continuous lines and primary cultures (Cenni et al., 1999).

Considering the concrete case of dental materials, different type of cells are used, such as human pulp cells (HPC) (Annunziata et al, 2005; Bolland et al., 2006; Spagnuolo et al., 2004; Stanislawski et al., 1999), dental papilla cells (Thonemann et al., 2002), human gingival fibroblasts (HGF) (Annunziata et al, 2005; Englemann et al., 2002; Issa et al., 2004; Moharamzadeh et al., 2006; Uo et al., 2003; Zao et al., 2004), dontoblast cells (Aranha et al., 2006; Bolland et al., 2006), and mouse fibroblast cells (L2929) (Cao et al., 2005; Franz et al., 2003; Messer et al., 2003; Robrta et al., 2003; Thonemann et al., 2002). Those are the prime target cells concerning dental restorative materials. But previous studies have already shown that dental materials could be responsible for a diversity of nocive effects in human health, like skin irritation, eyes or mucous membranes and even gastrointestinal problems (Lonnroth, 1997; Mathias et al., 1987). Beside the problems above, particles of those materials (≤10μm) can be inhaled and cause inflammation at lung's rabbit (Goldberg, 1992). So, studies involving rat alveolar epithelial and alveolar macrophages are done (Becher et al., 2006; Reichl et al., 2001), such as *in vitro* methodologies using keratinocytes (Moharamzadeh et al., 2006), relevant to predict those kind of reactions. Inflammatory response from the host tissue happens frequently, what request *in vitro* assays that include the use of cells which play an importante role concerning the response of tissue to biomaterial, monocyte and lymphocyte cell lines (Heil et al., 2002; Noda et al., 2003).

There's another point of interest that should be carefully analysed in the interpretation of data obtained in *in vitro* tests; the uptake of cells from different

species (non human), although easier to obtain, make the interpretation and extrapolation of data difficult (Kirkpatrick et al., 2002).

5. Analysed parameters

As mentioned in the previous section, there are different methods to determine biocompatibility, each one by the evaluation of different parameters. From the data collected in those methodologies it's possible to establish a cytotoxicity index (Hornez et al., 2002: Rogero et al., 2003), as well as other reactions from the organism, like the inflammatory response (Dee et al., 2002; Noda et al., 2003; Moharamzadeh et al., 2006).

The proteins behaviour is of great importance, because they are responsible for the interface between the tissue and the implant; after the implant, the first step is the adsorption of proteins to the surface of the biomaterial. The primary structure of proteins (amino acids sequence) controls the surface activity (Dee et al., 2002), as large the molecules are, major will be the probability of interactions with the surface, what is explained with the number of contact points between them. This turns that unfolding of the proteins stimule the adsorption process (process in which molecules adhere to solid surfaces), exactly by the same reason mentioned before, the unfolding enlarge the contact between surface-molecule. Thus, the layer formed by proteins-surface implant, is determinant to the cellular response to the medical device (Dee et al., 2002).

The first event after an implant being placed within the body is the inflammatory response; this process allways happen (with different degrees of intensity), because the implant procedure involves damage of tissue around, and the organism reaction to this is the inflammatory response. For situations like those, researchers study *in vitro* monocyte and lymphocyte functions. They release growth factors and cytokines around the implant, during the inflammatory process, manipulating tissue response to the implant (Page, 1991; Cenni et al., 1999; Dee et al., 2002; Noda et al., 2003). At the damaged tissue, macrophages and neutrophils remove dead cells, and the last one release proinflammatory cytokines, such as interleukins. Those are related to gingivitis and periodontal diseases (Moharamzadeh et al., 2006).

Cytotoxicity may be achieved by different methodologies that are responsible for the evaluation of cell function alterations or even death. So, there are functions

common to all cells that can be evaluated by cytotoxicity tests, such as: enzyme activity (Annunziata et al., 2005; Aranha et al., 2006; Boland et al., 2006; Holtz et al., 2005; Uo et al., 2003), cell viability (Cao et al., 2005; Fotakis and Timbrell, 2006; Rogero et al., 2003) or cell proliferation (Fotakis and Timbrell, 2006; Reichl et al. 2001; Uo et al., 2003).

6. Evaluation methods

Biocompatibility tests are imprescendible studies respecting to a medical device. There are a range of methods used to test cytotoxicity and other situations observed during a biocompatibility study, as mentioned above. For the evaluation of all the phenomenons mentioned in the previous section, there are different methodologies that are going to be summarized in Table 1.

Table 1: Summary of methodologies used in *in vitro* biocompatibility tests.

Tests	Function	References
Neutral Red	Cellular viability	• Rogero et al., 2003 • Cao et al., 2005 • Fotakis and Timbrell, 2006
ELISA	Inflammatory response	• Heil et al., 2002 • Noda et al., 2003 • Moharamzadeh et al., 2006
Flow cytometry	Number cell count	• Franz et al., 2003 • Spagnuolo et al., 2004
Alamar blue	Metabolic activity	• Uo et al., 2003 • Holtz et al., 2005
LDH	Cell membrane damage	• Reichl et al., 2001 • Issa et al., 2004 • Fotakis and Timbrell, 2006
Glutathione	Viability and concentration of intracellular agent glutathione	• Englemann et al., 2002
Microscopic observation	Count cells; Cell morphologie	• Franz et al., 2003

		• Theiszová et al., 2005
Fluorometry	DNA quantitation	• Uo et al., 2003
Bradford method	Protein measurement	• Reichl et al., 2001 • Fotakis and Timbrell, 2006
MTT	Mitochondrial activity	• Stanislawski et al., 1999 • Sjögren et al., 2000 • Schmalz et al., 2002 • Thonemann et al., 2002 • Roberta et al., 2003 • Issa et al., 2004 • Zhao et al., 2004 • Annunziata et al., 2005 • Holst et al., 2005 • Ronald et al., 2005 • Saw et al., 2005 • Theiszová et al., 2005 • Aranha et al., 2006 • Becher et al., 2006 • Boland et al., 2006 • Fotakis and Timbrell, 2006

Different sensibilities are detected in cell cultures to the tested toxics, depending on the cytotoxicity test used (Tanaka et al., 1998; Theiszová et al., 2005; Fotakis and Timbrell, 2006). This discrepance profiles might be due to differences in the methodologie applied in each assay. LDH, neutral red and, undoubtedly, MTT are the most used assays to predict cytotoxicity (Fotakis and Timbrell, 2006). LDH leakage assay consists in the release of the enzyme - lactate dehydrogenase, beyond damage membrane into the culture medium, with *in vitro* LDH release is possible to measure precisely the cell membrane integrity and consequently cell viability (Fotakis and Timbrell, 2006). In neutral red assay occurs the measure of the uptake dye, by lysosomes of viable cells (Fotakis and Timbrell, 2006). The MTT assay is based on the enzymatic conversion of a tetrazolium salt (MTT)

by succinate dehidrogenase in the mitochondria. The reduction mentioned only take place when mitochondrial reductase enzymes are active, which allows to associate the conversion to the number of viable cells (Ronald et al., 2005; Saw et al., 2005; Schmalz et al., 2002; Sjögren et al., 2000). Although of the differences observed, *in vitro* cytotoxicity assays are widely used to determine human toxicity from medical devices (Scheers et al., 2001). It has already been observed, like mentioned above, that different cytotoxicity assays turns different results concerning to their toxicity, and this is probably dependent of the agent in study and the differences in the methodologie (Weyermann et al., 2005). The uptake mechanisms of the cell lines relatively to the component in study must be analysed (Fotakis and Timbrell, 2006). ELISA technique is mentioned in Table 1 because the inflammatory response is always present when an implant is placed; thus, ELISA is of great importance in the quantitation of inflamatory mediators released (Kirkpatrick et al., 2002). All the mentioned assays are used in large scale in biocompatibility assays, although the MTT assay appears more commonly in cytotoxicty tests, what could be attributed to the methodologie simplicity.

7. Future developments

In vitro methologies present limitations caused by several factors, like differences between different species cultivated, phenotypic alteration of cell cultures as time passing by, loss of 3D organization and the difficult to associate data with the *in vivo* situation (Dee et al., 2002). Systems of cell cultures are, generally, constituted by one cell type, excluding the occurrence of a natural defense mechanisms promoted by the immune system or possible interactions with other cell types (Hornez et al., 2002). Thus, co-cultures can't be forgotten, because *in vivo* there is all kind of interactions between different cell types, influence the contact cell-material. So, the study *in vitro* of cell-cell interactions allows predicting the implant success (Dee et al., 2002; Wei et al., 2006). Considering this, development should point to minimize this kind of issue, that undoubtly affect the results obtained using those cell cultures (Kirkpatrick et al., 2002; Wei et al., 2006). The building of 3D structures will be able to solve the problem related to the proximity with the real situation, considering the fact that our tissues are 3D structures (Tan and Desai, 2004), as well as the perception of angiogenesis process represent a major development (Kirkpatrick et al., 2005), because the formation of new blood vessels is

fundamental to guarantee the survival of those formed tissues (Dee et al., 2002). The use of 3D culture systems is fundamental to better understand cell physiology and consequentely predict their behaviour when contacting prosthesis.

Stem cells are one of the perspectives of tissue engineering (Braccini et al., 2005; Dee et al., 2002; Drukker and Benvenisty, 2004). Until today, the use of those cells involve a lot of bioetical problems, difficult to resolve, imparing their use in *in vitro* tests. The use of primary cells or cell lines should be further discussed in the future, because there are results pointing to differences in the phenotypes expressed *in vitro* between them, and between each cell lines (Unger et al., 2004). At present, therapies of substitution using stem cells consist in a first step where cells are induced to differentiate to specific cells, desired to the concret problem, and in a second phase those cells are transplanted into a patient to replace the target tissue. One of the first goals of regenerative medicine is the establishment of a universal cell-line, what allows their transplantation to anyone (Drukker and Benvenisty, 2004).

The fact of tissue engineering is a recent area of study makes difficult to establish boundaries between wrong or right because legislation is not complete as it has serious difficulties in following such an expansive field. Maybe promissory results achieve investiment that allow larger development. In the future it should be possible to proceed to the remotion of organ-specific cells from diseases patients, which would be genetically manipulated *in vitro*, and located within again allowing the development of a "mosaic tissue", or tissue with his own diseased cells, in addition to his personal genetically repaired cells (Vacanti, 2006).

8. Conclusion

Being the study of biocompatibility such a multidisciplinary subject, its obvious the need of complement each science with all the others to achieve reasonable and relevant development in this field. The primordial role that cell cultures represent turns patent the require development of tissue engineering, because the future probably will be the "reparation" of body pieces, from regeneration and reconstruction. While this is not possible, the necessity of manufacturing more biocompatible and biofunctional materials remains. For that, cytotoxicitiy tests represent a huge help in the study of materials biocompatibility, being standardized procedures as much as possible. However, technology development is an ongoing process, what turns standardization in a difficult process to achieve. In spite of the great advances verified in biocompatibility tests in last years, the existence of some gaps respecting to environments *in vitro* more similar to the ones observed *in vivo* was notorious. This should be a concern for tissue engineering, but obviously not just this science is needed for the resolution of such a complex problem. Like mentioned before, the intercommunication between all the areas involved in biomaterials manufacturing and placement is fundamental to understand what exactly is needed.

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Capítulo III

Toxicity of Zirconium on growth of two green algae

Toxicity of Zirconium on growth of two green algae

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Abstract

Due to the growing application of zirconia in the manufacturing of prosthesis, and adding to the fact those being placed every day in numerous dentistry clinics, it became relevant to understand in which way the presence of zirconium (Zr IV) in aquatic environments is responsible for causing environmental changes.

The potential toxicity effect of Zr (IV) was tested using two green algae: *Pseudokirchneriella subcapitata* (Korshikov) Hindak and *Pandorina morum* (Müller) Bory. Algae cultures were grown at four different values of pH (6.5; 7.2; 7.5 and 8.0) in the presence seven different concentrations of the compound.

Concerning the toxicity elicited by zirconium on the green algae tested, this metal inhibited growth of both, with *P. morum* being more sensitive. Furthermore on alkaline environment seems to promote the toxic effects of zirconium.

Keywords: Zirconium, Growth inhibition, *Pseudokirchneriella subcapitata*, *Pandorina morum*, IC_{50}.

1. Introduction

When pollutants are released into aquatic habitats, there are almost always direct effects on aquatic biota. Direct effects depend on the intensity and duration of exposure to the toxic (Long et al., 1995), and they usually reduce organism abundance either by increasing mortality or reducing fecundity (Fleeger et al., 2003).

Their bioavailability and probable toxicity are regulated by metal speciation in water (Meylan et al., 2003). Parameters such as pH, redox potential, temperature, ionic content, mineral particles, organic matter and light exposure influence the metal chemical form and as a result its availability (de Filippis and Pallagly, 1994; Jjemba, 2002; Thiele-Bruhn, 2003; Tolls, 2001). The uptake of some metals by microalgae has been reported as being sensitive to pH alterations (Boullemant et al., 2004; Martínez and McBride, 2001).

Environmental contamination with metals from exceeding biomaterials used in dentistry can be considered at present as an environmental problem. For this fact has contributed the technology advances that turned the implant placement into a relatively simple technique.

Zirconia (ZrO_2) is a zirconium oxide, an inert bioceramic that show an increase, concerning to its application since 1993 when it started to be used in dental implants (Li and Hastings, 1998). This fact can be ascribed to its mechanical and physical properties, namely its great biocompatibility and inertia in a physiological environment. The studied compound was the metal zirconium. Zirconium is a chemical element, assigned with the symbol Zr, resembling titanium. It is a white-grayish metal, lustrous and exceptionally resistant to corrosion. Zirconium is lighter than steel and its hardness is similar to copper. Its principal source is the zirconium silicate mineral, zircon ($ZrSiO_4$), which is found in deposits located in Australia, Brazil, India, Russia, and the U.S.A.. Considering the transition elements only Fe, Ti and Mn are more abundant than zirconium, which comprise 0.016% (162 ppm) of the earth's crustal rocks (Greenwood and Earnshaw, 1984; Hulbert, 1993; Li and Hastings, 1998).

In spite of the several studies surrounding the aim of this study (Kolpin et al., 2002; Ternes, 1998; Ternes et al., 2002; Kümmerer et al, 2000), none of them was related to the use of biomaterials, despite the exponential growth of their application. This kind of material is manufactured objectively for a location inside the human body, but it is conceivable that it can also elicit biochemical and physiological changes in aquatic

environment due to its large use and residues released into the environment (Jjemba, 2006). During sewage treatments, most of the compounds released into aquatic environments, are not removed and remain in the effluents that get to the surface and groundwater (Doll and Frimmel, 2003; Ternes, 1998; Möhle et al., 1999). Because any effect on the lowest level of the food chain will also have consequences on the other trophic levels, algae are very suitable organisms for the determination of the impact of toxic substances on the aquatic environment (Joubert, 1980).

In the present work, two microalgae species have been used in order to test the toxic effect of different concentrations of zirconium (Zr IV) and pH influence on Zr (IV) toxicity was also tested.

2. Material and Methods

Since the first tests started (Doudodoroff et al., 1951; Hart et al., 1945), researchers have been working to prepare more refined tests in which organisms of several trophic levels of the food chain were used (APHA, 1992).

At present, and from a few years ago, algae, and particularly unicelular *Selenastrum capricornutum*, become a constant presence in biological tests (USEPA, 2002). *Selenastrum capricornutum* is the formerly name gave to *Pseudokirchneriella subcapitata* (Korshikov) Hindak, and usually used as standard species for algae toxicity tests (ASTM, 2002; OECD, 2002; USEPA, 2002).

In this study *Pandorina morum* and *Pseudokirchneriella subcapitata*, both green algae, have been chosen to test the effects of zirconium over their growth. *Pandorina morum* (Müller) Bory was obtained in the environment and isolated by micromanipulation in laboratory, with a micropipette under a light microscope, pre-cultured at least one month in MBL medium in a cabinet (F10 000 EDTU model) and maintained in aseptic conditions. *Pseudokirchneriella subcapitata* (Korshikov) Hindak was purchased from Alga-Gro® Freshwater, Carolina Biological Supply Company – Burlington, North Carolina 27215.

The nutritive culture medium used was the Marine Biological Laboratory (MBL) (Stein, 1973). To prepare 1 L MBL medium, sterile distilled water was used in the preparation of medium to which stock solutions were added: 1 mL of $CaCl_2.2H_2O$ ($36.76g.L^{-1}$); $MgSO_4.7H_2O$ ($36.97g.L^{-1}$); $NaHCO_3$ ($12.60g.L^{-1}$); K_2HPO_4 ($8.71g.L^{-1}$);

$NaNO_3$ (85.01g.L^{-1}); $Na_2SiO_3.9H_2O$ (28.42g.L^{-1}); Na_2.EDTA (4.36g.L^{-1}); $FeCl_3.6H_2O$ (3.15g.L^{-1}); $CuSO_4.5H_2O$ (0.01g.L^{-1}); $ZnSO_4.7H_2O$ (0.022g.L^{-1}); $CoCl_2.6H_2O$ (0.01g.L^{-1}); $MnCl_2.4H_2O$ (0.18g.L^{-1}); $Na_2MoO_4.2H_2O$ (0.006g.L^{-1}) and 2 mL of Tris(hydroxymethyl-)-amino-methane (50g.200mL^{-1}). Vitamins (previously sterilized by filtration) were added only after MBL sterilisation.

Effects of zirconium (Zr IV) were studied using the compound $ZrO(NO_3)_2.xH_2O$ (Sigma-Aldrich, 380679 18411BB). In preliminary tests, it was certified that the presence of nitrates didn't affect algae growth, by growing them for the same 96 h in the presence of the amount of nitrates present in the compound used. The compound was diluted in MBL and tested in different concentrations (0.00; 0.50; 2.00; 4.00; 6.00; 8.00 and 10.00mg.L^{-1}) previously estimated in preliminary tests. For all concentration experiments were also carried out at four different pH values – pH 6.5, 7.2, pH 7.7 and pH 8.0 (pH adjusted with HNO_3^- slowly added to the sample).

Cultures were grown in 250 mL Erlenmeyer flasks with 100 mL of synthetic culture medium. A sample of each freshwater green algae was placed in 100 mL Erlenmeyer flasks with 40 mL of final test volume (Gonçalves et al., 2005). To each test conditions suited a set of three replicates was performed. An inoculum culture was incubated under the same conditions as the test cultures, 3 or 4 days before the test started. Inoculates were obtained from exponentially growing cultures. The initial cell concentration used for the green algae was about $5x10^4$ cells.mL^{-1}. Toxicity tests were conducted for a period of 96h in the same conditions as those described above for the algal maintenance procedure, in 100 mL Erlenmeyer flasks containing 40 mL of synthetic culture medium. At the end of the 96h three biomass parameters were evaluated: optical density at 440 nm, cell counting and chlorophyll *a* (chl a) concentration. Cell counting was achieved using a Newbauer chamber for *P. subcapitata* and Sedgwick-Rafter chamber for *P. morum,* previously immobilized with Lugol (APHA, 1992). A 6505 UV/VIS spectrophotometer (JENWAY) was used to measure optical density at 440 nm. Chlorophyll *a* (chl *a*) concentration was determined by filtering the remaining culture (approximately 20 mL) through Whatman GF/C filters, which were then treated with acetone (90%) to extract the chl *a* that was measured at 665 nm and 750 nm, before and after acidification with HCl (0.1 M) (Jeffrey and Humphrey, 1975).

The results obtained with the different concentrations of zirconium (Zr IV) tested were compared using one-way analysis of variance (ANOVA). The results of toxicity tests based on the growth inhibition of the algae were reported as IC_{50}. These values were calculated for the different species using the Probit analysis (Finney, 1971).

After one-way ANOVA, a multiple comparison test (Tukey´s *t* test) was applied, when applicable (Zar, 1996; Sokal and Rohlf, 1995). Significant differences in growth were reported for $p<0.05$.

3. Results

After analysis, data obtained from the various tests under the same conditions showed the absence of a significant difference between our control (CTL) and the first concentration tested (0.5 $mg.L^{-1}$), for all the pH values (pH 6.5; pH 7.2; pH 7.5 and pH 8.0) tested: CTL and 0.5 $mg.L^{-1}$ of Zr IV, allowed growth of *P. subcapitata* and *P. morum*, with no significant differences. Therefore, it was assumed 0.5 $mg.L^{-1}$ as control to establish comparisons. This fact can be observed in Figure 1 and Figure 2, for *P. morum* (ANOVA, $p<0.05$) and *P. subcapitata* (ANOVA, $p<0.05$), respectively.

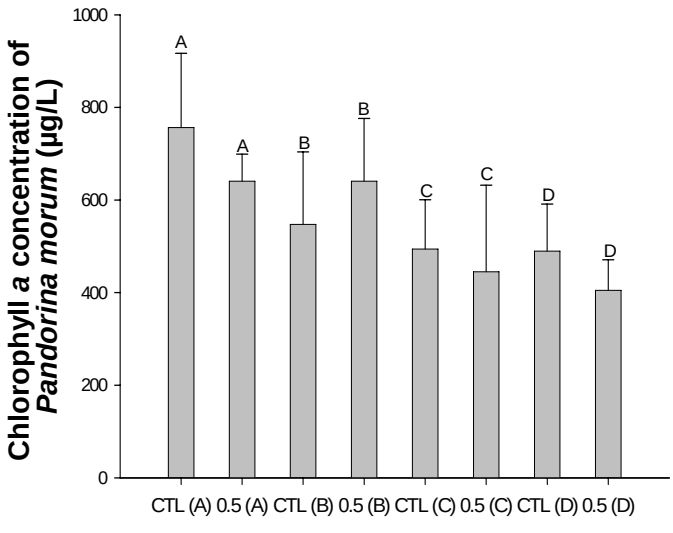

Figure 1: Algal growth, at different concentrations of Zr (IV), after 96h of incubation using *P. morum* at (A) pH 6.5, (B) pH 7.2, (C) pH 7.5 and (D) pH 8.0. CTL correspond to 0.00 mg/L and 0.5 to 0.5 mg/L of Zr. Data are the mean of 3 replicates and error bars represent the standard deviation.

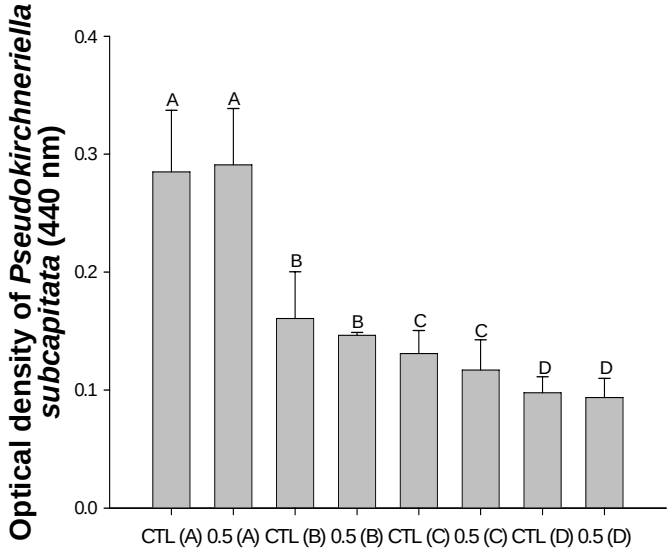

Figure 2: Algal growth, at different concentrations of Zr (IV), after 96h of incubation using *P. subcapitata* at (A) pH 6.5, (B) pH 7.2, (C) pH 7.5 and (D) pH 8.0. CTL correspond to 0.00 mg/L and 0.5 to 0.5 mg/L of Zr. Data are the mean of 3 replicates and error bars represent the standard deviation.

The growth curves obtained for *P. morum* (Figure 3) and *P. subcapitata* (Figure 4) after 96h of incubation show that *P. morum* presented a higher sensitivity to the compound tested relatively to *P. subcapitata.* In the same figures the influence of pH on toxicity can be observed: as pH increases, a considerable decrease in the growth occurred (a decrease that was bigger to *P. morum).*

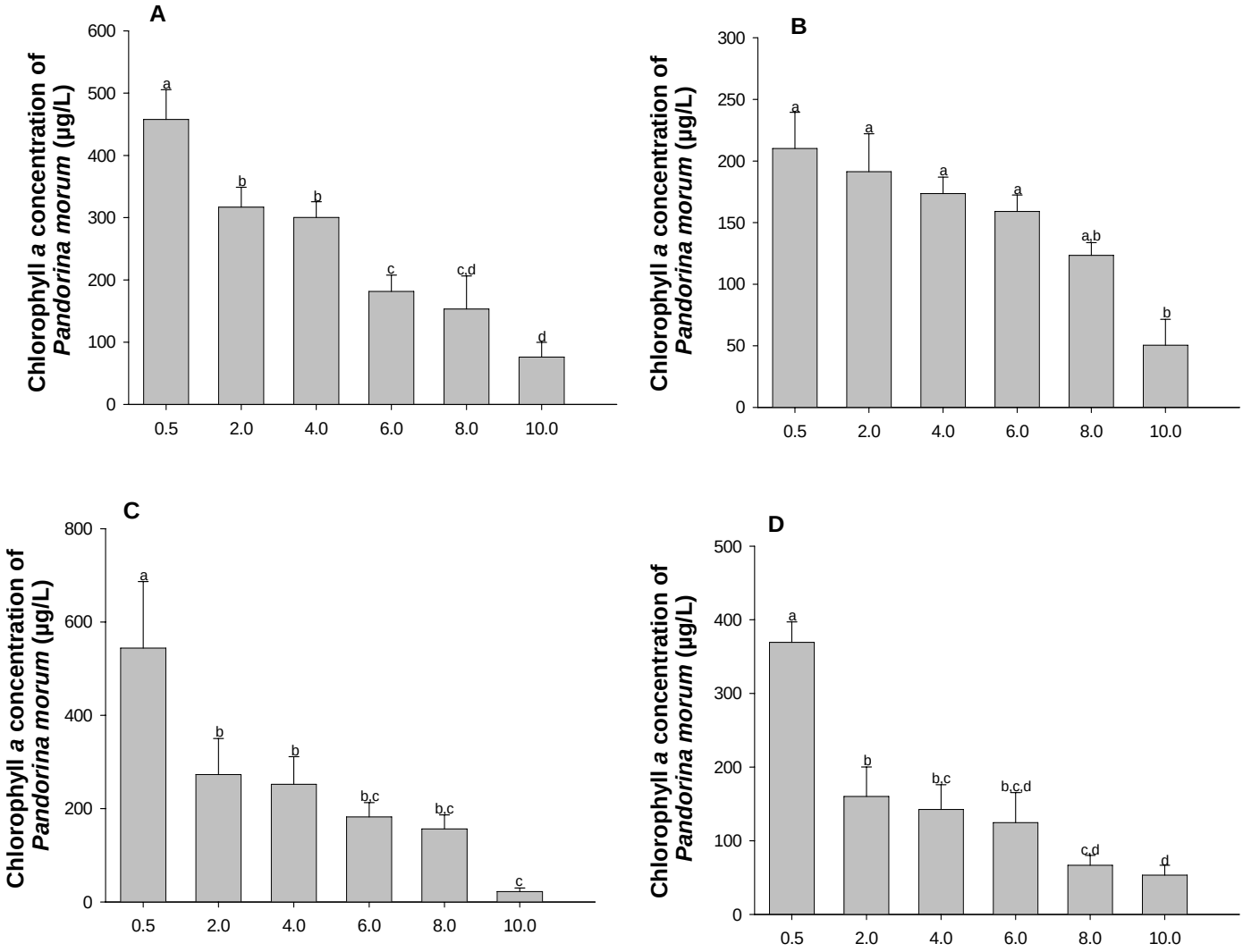

Figure 3: Algal growth, at different concentrations of Zr, after 96h of incubation using *P. morum* at (A) pH 6.5, (B) pH 7.2, (C) pH 7.5 and (D) pH 8.0. Zirconium concentrations tested were 0.50 mg/L; 2 mg/L; 4 mg/L; 6 mg/L; 8 mg/L and 10 mg/L. Data are the mean of 3 replicates and error bars represent the standard deviation. The different letters

correspond to significant differences between the treatments ($p < 0.05$). The absence of letters means there are no significant differences among all the treatments.

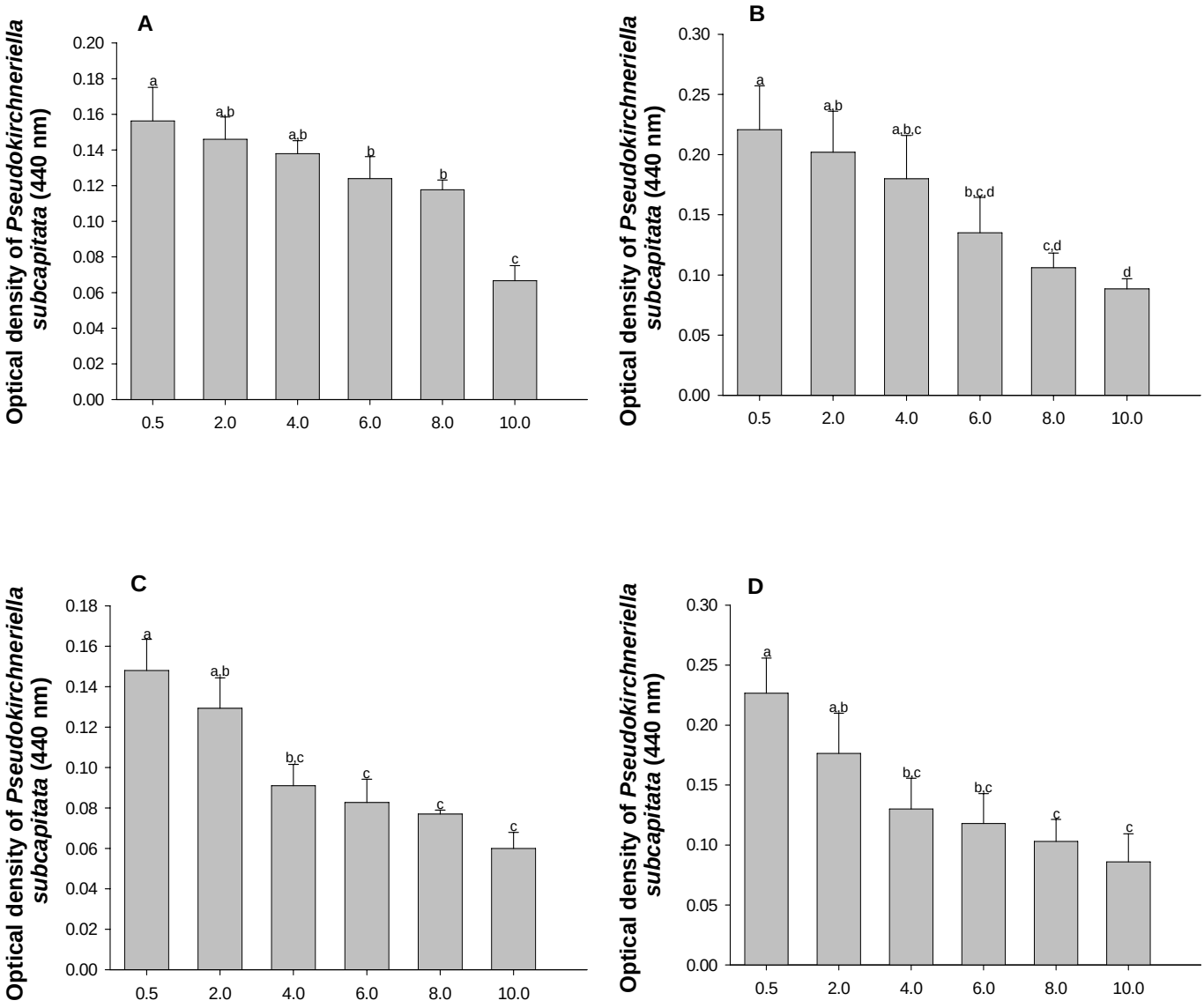

Figure 4: Algal growth, at different concentrations of Zr, after 96h of incubation using *P. subcapitata* at (A) pH 6.5, (B) pH 7.2, (C) pH 7.5 and (D) pH 8.0. Zirconium concentrations tested were 0.50 mg/L; 2 mg/L; 4 mg/L; 6 mg/L; 8 mg/L and 10 mg/L. Data are the mean of 3 replicates and error bars represent the standard deviation. The different letters correspond to significant differences between the treatments ($p < 0.05$). The absence of letters means there are no significant differences among all the treatments.

For *P. morum*, significant differences in growth (comparing to growth in the presence of 0.5 $mg.L^{-1}$Zr IV) were found for different concentrations, depending on pH value considered. For pH 6.5 significant differences occurred for concentrations ≥ 6 $mg.L^{-1}$, for pH 7.2 concentrations ≥ 10 $mg.L^{-1}$ and at last pH 7.5 and pH 8.0 revealed significant differences for concentrations ≥ 2 $mg.L^{-1}$ (Figure 3). Concerning *P. subcapitata* (comparing with control), significant growth inhibition was observed for concentrations ≥ 6 $mg.L^{-1}$, for pH 6.5 and pH 7.2, concentrations ≥ 4 $mg.L^{-1}$ for pH 7.5 and to pH 8.0 an inhibitory effect was felt for concentrations ≥ 2 $mg.L^{-1}$ (Figure 4).

Table 1 presents the median effective concentration at 50% growth inhibition (IC_{50}) for the different concentrations and pH values tested. Figures 3 and 4 and Table 1 led to the conclusion that an inhibitory effect caused by the increase of zirconium concentration exist for both green algae used, but the toxicity is higher for *P. morum*, considering the same concentrations of zirconium.

Table 1: Summary of the results ($96IC_{50}$) from growth inhibition tests of *Pseudokirchneriella subcapitata* (Korshikov) Hindak and *Pandorina morum* after exposure to the toxic compound.

Species tested / **pH values**	***P. morum*** **IC_{50} ($mg.L^{-1}$)**	***P. subcapitata*** **IC_{50} ($mg.L^{-1}$)**
6.5	**4.6 (3.2 – 6.5)***	**9.8 (8.4 – 12.7)***
7.2	**8.1 (6.9 – 9.8)***	**7.9 (7.1 – 9.1)***
7.5	**3.2 (1.2 – 5.4)***	**7.3 (6.3 – 8.6)***
8.0	**2.9 (1.2 – 4.9)***	**6.2 (5.4 – 7.3)***

* Corresponding to 95% confidence intervals

Regarding *P. morum*, the IC_{50} were 4.6 $mg.L^{-1}$ (95% confidence limits of 3.2 – 6.5), 8.1 $mg.L^{-1}$ (95% confidence limits of 6.9 – 9.8), 3.2 $mg.L^{-1}$ (95% confidence limits of 1.2 – 5.4) and 2.9 $mg.L^{-1}$ (95% confidence limits of 1.2 – 4.9) for pH 6.5, pH 7.2, pH 7.5 and pH 8.0 respectively. For *P. subcapitata* these values were 9.8 $mg.L^{-1}$ (95% confidence limits of 8.4 – 12.7), 7.9 $mg.L^{-1}$ (95% confidence limits of 7.1 – 9.1), 7.3 $mg.L^{-1}$ (95% confidence limits of 6.3 – 8.6) and 6.2 $mg.L^{-1}$ (95% confidence limits of 5.4 – 7.3) respecting the same order mentioned to *P. morum*.

4. Discussion and Conclusion

The Zr IV toxicity displayed by the two algae used was significantly different. The differences observed can be ascribed to the different degrees of sensitivity revealed by them, as observed by Pereira et al.(2005). Therefore, *P. morum* proved to be more sensitive (presenting lower values of 96h IC_{50}) than *P. sucapitata* to Zr IV exposure. *P. sucapitata* showed to be tolerant more than twice the IC_{50} concentrations obtained for *P. morum*. Therefore, according to the results obtained, reports and literature, researchers should be aware of interspecific differences concerning sensibility when evaluating heavy metals toxicity towards microalgae (Rojíčková and Maršalek, 1999; Yan and Pan, 2002).

Several studies have already revealed the influence of pH on the toxicity of metals to microbiota: metal toxicity can either increase or decrease with the pH alterations. Alterations of pH can influence some aspects of the cell-metal system, such as the metabolic state of the cell (with possible physiologic alterations) or chemical speciation of metals (*e.g.* in seawater of pH 8.5, Pb occurs as $PbOH^+$, Zn as $Zn(OH)_2$ (Hahne and Kroontje, 1973), Cu as $Cu(OH)_2$ (Zirino and Yamamoto, 1972), while in acidic lake waters, all the metals above occur as divalent cations. The different speciation forms of the same metal can determine different degrees of toxicity. Considering the same algae used in the present study (*Pseudokirchneriella subcapitata)*, other authors showed that increasing the pH of the medium from acidic to alkaline levels, promotes lower levels of toxicity of Pb to the species above (Monahan, 1976), in opposition to results concerning Zr (IV). Studies involving copper, cadmium and zinc suggests that increasing pH values results in an increase of metal toxicity (Borgmann, 1983) and Martínez and McBride (2001), using the same metal species concluded that higher pH promotes the potential availability of the

co-precipitates. These results are in agreement with those obtained in the present study: increase of Zr (IV) toxicity with pH increase, as observed by De Schamphelaere et al. (2003), using copper.

According to the results obtained for the growth inhibition of *Pseudokirchneriella subcapitata* (Korshikov) Hindak and *Pandorina morum* (Müller) Bory caused by zirconium, the present study shows that the biomaterial inhibits the growth of the two green algae. The toxic effect was observed for the two species, but stronger to *P. morum.* The toxicity was observed with more intensity in alkaline environments. Increases of pH value stimulate the compound toxic effect, considering the same concentration.

Data of this kind of wastes in environment point to relatively low concentrations, on the nanogram and microgram ranges, concentrations that are unlikely to elicit severe toxicity (Daughton and Ternes, 1999; Ferrari et al., 2003). The occurrence of biomaterials in the environment is nevertheless worrying because, although they are usually at such low concentrations, they are continuously being discharged into a receiving aquatic system. This circumstance presents a continued exposure of these compounds to organisms. Future work should contemplate the risks considering the cumulative effects (chronic toxicity) instead of just the once acute toxicity assay (Laskowski, 2001) and studies should be design to address the possible toxicological effects of such low, but continued exposure.

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Capítulo IV

Discussão geral

Discussão geral

Após a análise cuidada de inúmeros artigos, foi possível observar e confirmar a grande complexidade que envolve a aplicação de biomateriais, nomeadamente a variedade de ciências que se coadunam para a consecução do objectivo final que é, a elaboração de dispositivos médicos que se assemelhem, tanto quanto possível, ao tecido ou órgão que pretendem substituir, no fundo pretende-se conseguir mimetizar a sua função. No entanto, e porque o nosso organismo se caracteriza por uma dinâmica intensa, nem sempre é possível prever as suas reacções a um objecto estranho, como o é um implante. Com o objectivo de minimizar estes efeitos indesejados, existem testes de biocompatibilidade, que são de realização obrigatória antes que qualquer dispositvo possa ser usado clinicamente. Após a análise de algumas das metodologias *in vitro*, no que respeita à biocompatiiblidade de materiais dentários, podemos concluir que a realização de testes de citotoxicidade são o primeiro passo de enorme relevância para os passos seguintes pelos quais o biomaterial terá que passar, pelo facto de a detecção de citotoxicidade ter, geralmente, carácter eliminatório. Pôde-se então verificar, da bibliografia citada, que a maioria dos testes de biocompatibilidade *in vitro* recorrem a testes de citotoxicidade, de resto como recomendado pela ISO 10993-5. Observou-se ainda, dentro da avaliação da citotoxicidade, a prevalência de testes de carácter qualitativo e quantitativo, fazendo parte dos primeiros a detecção de alterações morfológicas na célula (por observação microscópica) que indiciem a existência de toxicidade (Dee et al., 2002). Dos métodos de análise quantitativa fazem parte a quantificação da morte celular e proliferação celular (Hornez et al., 2002; Rogero et al., 2003). Assim, a avaliação da citotoxicidade recorre à realização de inúmeros métodos, mencionados na Tabela 1 (Capítulo II), que consistem na observação e medição de alterações das funções celulares, provocadas pela toxicidade do material em estudo (ISO 10993-5). Após a análise da Tabela 1 (Capítulo II), facilmente se concluiu que o método MTT é sem dúvida o mais requisitado para a realização de testes de citotoxicidade. A preferência dever-se-á possivelmente às características observadas na descrição do teste (Capítulo II, secção 5), tais como a rapidez e facilidade da metodologia em questão, bem como a precisão revelada pelo método. A ausência de radioisótopos é também uma mais valia (Mosmann, 1983).

Embora os avanços relativamente aos testes de biocompatibilidade sejam indiscutíveis, é possível denotar a existência de algumas lacunas, nomeadamente no que respeita à

necessidade de desenvolvimento da prática de culturas celulares, concretamente a necessidade de construir estruturas 3D, que irão aproximar os modelos *in vitro* da realidade do nosso organismo, tornando desta forma muito mais simples extrapolar os resultados obtidos para a prática clínica (Tan and Desai, 2004). Para isto muito contribuirá também o uso de células estaminais, que sendo provenientes do organismo do paciente, permitem transpôr a grande barreira que representa o sistema imunitário, não obstante os inúmeros estudos que lhe têm sido dedicados (Heil et al., 2002; Noda et al., 2003; Moharamzadeh et al., 2006). Compete à Engenharia de Tecidos conseguir encontrar respostas para estes problemas. Tratando-se de uma área recente é no entanto complicada a sua consolidação, nomeadamente no que respeita à ausência de legislação e a todos os problemas éticos que a envolvem. Por outro lado, os encargos que comporta não são facilmente suportáveis, sendo que as seguradoras não cobrem ainda este tipo de tratamentos, por se considerar que existem tratamentos tradicionais que, melhor ou pior, conseguem remediar a situação. Apesar dos inúmeros progressos que esta ciência tem conhecido, não possui ainda muita aplicação humana (Vacanti, 2006).

Relativamente à parte experimental, que consistiu na avaliação da ecotoxicidade do Zr (IV) no que respeita às duas algas verdes em estudo - *Pseudokirchneriella subcapitata* (Korshikov) Hindak e *Pandorina morum* (Müller) Bory, concluiu-se que o crescimento de ambas é afectado pela presença do metal, verificando-se, de acordo com os resultados obtidos, que *P. morum* é mais sensível que *P. subcapitata* (Capítulo III, Figuras 3 e 4), o que confirma resultados obtidos em estudos anteriores, que referem a existência de diferentes graus de sensibilidade de acordo com a espécie em estudo (Pereira et al., 2005). O metal apresenta assim diferentes níveis de toxicidade relativamente às duas espécies, o que se verificou pelos valores de CE_{50} determinados (Tabela 1, Capítulo III). Pela análise destes é possível verificar que *P. subcapitata* manifesta uma tolerância cerca de duas ordens de grandeza superior aquela verificada para *P. morum*. Ainda no que respeita à toxicidade manifestada por Zr (IV), observou-se que esta é influenciada pelo pH do meio; assim, a toxicidade é superior em meios alcalinos, tendo as duas algas respondido de igual forma no que respeita a este parâmetro em análise. No que se refere à influência do pH na toxicidade de metais, esta foi já vastamente estudada relativamente à sua influência em metais pesados, resultantes em grande parte da erosão natural e da actividade humana (de Filippis and Pallaghy, 1994; Wei et al., 2003), sendo que o estudo não incluiu nunca metais

utilizados na concepção de biomateriais, como o abordado neste estudo. Características físico-químicas do meio como o pH, potencial redox, composição iónica, partículas minerais, conteúdo em matéria orgânica, temperatura ou luz, influenciam a forma química, a mobilidade e a biodisponibilidade dos elementos ou substâncias, bem como dos respectivos efeitos tóxicos (Babich & Stotzky, 1980). A toxicidade dos metais relativamente às algas, quer seja individual ou combinada, depende fundamentalmente do tipo e concentração do metal, do pH do meio e da presença de ligandos naturais ou artificiais (EDTA, ácido cítrico, ácido glicólico) (Starodub et al., 1987). Após a análise de diversos estudos relativos à influência do pH na toxicidade de metais, pôde assim concluir-se que não existe uma regra comum, havendo resultados completamente díspares dependendo do metal que estejamos a estudar e das condições em que este se encontra. No entanto, pelo aumento verificado na sua utilização e pela ausência de estudos nesse sentido, é importante reforçar a constatação que resultou deste estudo, que nos permitiu concluir que, no caso do Zr (IV) existe um aumento nítido da sua toxicidade resultante do aumento do pH do meio.

Pretendeu-se com este trabalho alertar para a ausência de bibliografia no que respeita a esta temática; embora os estudos de ecotoxicidade abundem, o mesmo não se verifica quando o agente em estudo é um biomaterial. No entanto, considerando o aumento na aplicação diária de biomateriais, é fundamental considerar os possíveis efeitos nocivos que estes poderão representar para o ambiente, quando não são devidamente tratados.

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